Organizing sites for regulated secretion in adrenal chromaffin cells

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CHAPTER 1

Introduction

Regulated secretion in adrenal chromaffin cells

Chromaffin cells: a general introduction

Chromaffin cells (CC) are neuroendocrine cells, which are localized in the medulla of the adrenal gland. CCs are (like sensory neurons of the peripheral nervous system) derivatives of the neural crest. Both sympathetic neurons and adrenal CCs develop from the same precursor cells, the so-called sympathoadrenal progenitors in the dorsal neural tube (Unsicker, 1993). What determines the fate of cells to become either sympathetic neurons or CCs is still unclear (Unsicker et al., 2013). Based on their common progenitor cells, a multitude of basic biological mechanisms is conserved in both systems. Besides neurons, CCs are the most used model to study regulated exocytosis. Secretory vesicles in CCs contain hormones and peptides such as catecholamines, which are released into the bloodstream upon depolarization (Bennett, 1941; Winkler et al., 1987; Unsicker, 1993). The life cycle of a secretory vesicle starts during vesicle biogenesis, which is followed by maturation processes and the transport to the destination location at the plasma membrane (PM), where secretory vesicles release their content in a highly regulated, stimulus-dependent manner (fig1.1).

Biogenesis of secretory vesicles in chromaffin cells

Precursor peptides of secretory neurotransmitters and hormones are synthesized at the rough endoplasmic reticulum (ER) and transported to the cisternae of the Golgi apparatus, where they are packaged into secretory vesicles. Short-lived immature secretory vesicles (ISVs) bud from the *trans*-Golgi network (TGN) and subsequently traverse several maturation steps to become mature secretory vesicles (MSVs). Newly generated ISVs are believed to be in an intermediate state with a half-live of ≈45 min (Tooze et al., 1991).

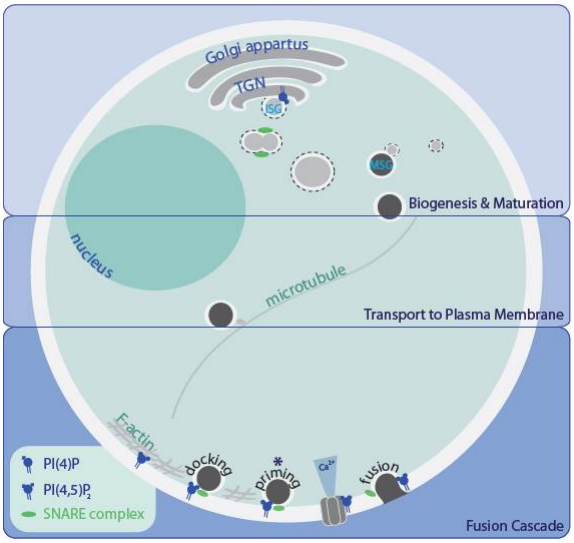

Figure 1.1: Schematic summary of regulated secretion in adrenal chromaffin cells: from biogenesis and maturation of secretory vesicles to exocytosis on the plasma membrane: Display of involvement of SNARE complexes (light green), lipids (blue) and cytoskeleton components (mintgreen/grey).

Furthermore, ISVs contain a distinct set of proteins which is not present in MSVs (Tooze et al., 1991). In order to remove ISV content and deliver MSV-specific molecules, a series of consecutive fusion and budding processes is necessary. Essential processes in the conversion of ISVs to MSVs are (1) the homotypic fusion of ISVs to mix content, (2) the acidification of the ISVs and (3) the content condensation by removal of constitutive secretory proteins and excess membrane (for reviews see (Kim et al., 2006), (Morvan and Tooze, 2008)).

Homotypic fusion of ISVs results in an increase in vesicle size during maturation (Tooze et al., 1991; Sombers et al., 2007). Regulatory roles in ISV-ISV fusion were reported for α-soluble N-ethylmaleimide sensitive fusion protein attachment protein (SNAP) and Syntaxin6 (Wendler et al., 2001) as well as Synaptotagmin-IV (Ahras et al., 2006). During the maturation of secretory vesicles the pH gradual decreases from the TGN to ISVs to MSVs. This is accomplished by an increase in amount and density of H^+ pumps on the vesicle membrane (Wu et al., 2001). Acidification is essential for the protein composition of the PM and the activation of enzymes (*e.g.*, prohormone convertases (Lindberg, 1991; Steiner et al., 1992) and carboxypeptidase E (Rindler, 1998)), which convert the precursor peptides into functional hormones and neurotransmitters (Colomer et al., 1996). Besides secreted proteins (*e.g.*, hormones and neurotransmitters), which are released in a regulated fashion, all ISVs contain accessory constitutive secretory proteins (Arvan et al., 1991), lysosomal enzymes (Kuliawat et al., 1997) and membrane-associated proteins.

This cargo, which is not targeted for regulated secretion, is removed by clathrin-dependent budding of constitutive-like vesicles from ISVs (Tooze and Tooze, 1986; Rosa et al., 1992). Hereby the core protein content of the maturing secretory vesicle is compressed and concentrated and excess membrane is removed. This membrane remodeling process was shown to be dependent on adaptor protein 1 (AP-1 (Dittie et al., 1997) and regulated by ADP ribosylation factor 1 (Arf1). Even though the maturation of ISVs in neuroendocrine cells was subject to a multitude of studies in the last 30 years, many mechanistic details are still unclear. In Chapter 2 of this thesis we describe a thus far unknown function of the SNARE protein Vti1a in the biogenesis of secretory vesicles in CCs.

Transport from the *trans*-Golgi network to release sites on the plasma membrane

The transport of secretrory vesicles in CCs depends on two components of the cytoskeleton: tracts of microtubules and a highly dynamic network of filamentous actin (F-actin) (for reviews see: (Trifaró et al., 2008; Papadopulos et al., 2013)). In CCs microtubules are concentrated in internal regions, especially around the Golgi apparatus, from where they radiate towards the periphery of the cell (Bader et al., 1981; Neco et al., 2003). In the cortical regions underneath the PM of CCs, the main component of the cytoskeleton is a dense F-actin network (Lee and Trifaró, 1981; Cheek and Burgoyne, 1986). In order to be transported from the Golgi apparatus to their release sites at the PM, secretory vesicles need to be able to interact with different components of the cytoskeleton. Secretory vesicles contain tubulin-binding sites (Bernier-Valentin et al., 1983) to connect with microtubules and binding sites for actin-binding proteins such as α-actinin (Jockusch et al., 1977; Aunis et al., 1980) and fodrin (Aunis and Perrin, 1984) which ensure the interaction between secretory vesicles and actin filaments. More recent studies focused on the function of myosin motor proteins in the transport of secretory vesicles (Neco et al., 2002). Myosin II and Myosin V were shown to be especially important in this process (Lejen et al., 2003). Besides its role as molecular motor, Myosin II is involved in cross-linking and bundling of actin fibers as well as actin polymerization and thereby plays an important role in regulated exocytosis in CCs (Neco et al., 2004; Bond et al., 2011). Myosin Va connects secretory vesicles to actin filaments via its interaction with Rab27 and MyRIP and regulates the spatial distribution of secretory vesicles (Desnos et al., 2003; Kögel et al., 2010). The network of cortical F-actin is suggested to function as a physical barrier for secretory vesicles approaching the PM (Vitale et al., 1991; Trifaró et al., 1992). Stimulation of CCs results in Ca^{2+} dependent de-polymerization of this F-actin network. Secretory vesicles gain access to release sites at the PM, where they become docked/tethered as first step of the fusion cascade.

Docking, priming, fusion

Regulated secretion of hormones and neurotransmitters is the most important function of neuroendocrine cells and fundamental to intercellular communication. Before a secretory vesicle fuses with the PM to release its content to the extracellular space, it needs to be in close proximity to the PM (dock) and gain fusion-competence (prime). The first necessary step during regulated exocytosis is the docking of secretory vesicles to the PM (for review see (Verhage and Sørensen, 2008)). According to the prevalent definition of morphological docking, the vesicle membrane and the PM must be in direct contact without any measurable distance between them. Docking was shown to be highly dependent on Munc18-1 (Voets et al., 2001), Syntaxin1a (de Wit et al., 2006), SNAP-25 and Synaptotagmin-1 (de Wit et al., 2009; Mohrmann et al., 2013), which are the molecular components that together form the minimal docking machinery. In addition to the proteins named above, the docking process in CCs is also regulated by the cortical F-actin network (Nakata and Hirokawa, 1992; Toonen et al., 2006). Furthermore, the PM localized phospholipid phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) was shown to be involved in the recruitment of vesicles to their release sites (Honigmann et al., 2013). After a secretory vesicle is docked to the PM, it needs to undergo the priming process in order to gain fusion-competence (for review see (Klenchin and Martin, 2000; James and Martin, 2013)). Important priming factors are Calcium-dependent activator protein for secretion (CAPS) (Jockusch et al., 2007) and Munc13 (Rosenmund et al., 2002; Ashery et al., 2000). More recent studies indicated the involvement of additional regulatory proteins in the priming process (Dulubova et al., 2005), such as RIM (Deng et al., 2011) and Rab3A (Schonn et al., 2010; Huang et al., 2011). $PI(4,5)P_2$ was also shown to be essential in the regulation of the vesicle priming process, since both CAPS and Munc13 interact with $PI(4,5)P_2$ (Kabachinski et al., 2014). Furthermore, diacylglycerol (DAG), a second messenger, generated by phospholipase C-mediated $PI(4,5)P_2$ hydrolysis was shown to bind and activate Munc13 (Ashery et al., 2000). The pool of primed vesicles on the PM is referred to as readily-releasable pool (RRP), since those vesicles fuse with the PM immediately upon Ca^{2+} influx. The final step of regulated exocytosis in CCs is the actual fusion of secretory vesicles with the PM. This process is initiated by an increase in the concentration of intracellular Ca^{2+} through the influx of Ca^{2+} ions through ion channels on the PM. The most important molecular components for fusion are the members of the SNARE complex (Syntaxin1a, SNAP-25 on the PM and synaptobrevin-2 on the membrane of the secretory vesicles (Söllner et al., 1993b; Bennett et al., 1992; Südhof et al., 1989). The assembly of those three proteins into a four-helical bundle results in the formation of a stable complex (*trans*-SNARE complex) between the PM and the vesicle membrane (Sutton et al., 1998). The membranes are forced together via a zippering mechanism of the complex, forming the *cis*-SNARE complex upon membrane fusion (Matos et al., 2003). At least three SNARE complexes are needed for regulated secretion of secretory vesicles (Mohrmann et al., 2010).

The fusion of both membranes and the release of the vesicle content into the extracellular space represent the end of the life of a secretory vesicle.

Even though the life of secretory vesicles is rather short and only contains a limited number of stages, it is incredibly complex. Scientific research in this field and on this cell type started already in the 1940s and we still do not completely understand all mechanisms in detail. Therefore, we tried to add some valuable insight and knowledge to at least two of the stages of the life of a secretory vesicle.

Cellular components involved in biogenesis and regulated exocytosis

SNARE complexes and Sec1/Munc-18 (SM) proteins

SNARE proteins and SM proteins represent the core machinery for intracellular membrane fusion processes. Most of what is known about SNARE proteins and SNARE complex formation originates from studies focusing on the molecular mechanisms of regulated exocytosis (for recent reviews see (Südhof and Rothman, 2009; Südhof, 2013)). All SNARE proteins are localized on membranes of cellular organelles and contain at least one α-helix. In order to generate the force to fuse membranes, three or four SNARE proteins form a complex, which is build up as a four-helical bundle with a coiled-coil structure (Sutton et al., 1998). The helices form a so-called SNARE-pin, which is subsequently zippered in N-to-C terminal direction (Walter et al., 2010). Besides SNARE proteins, SM proteins are considered key components of the membrane fusion machinery (Gallwitz and Jahn, 2003; Malsam et al., 2008; Südhof and Rothman, 2009). SM proteins directly interact with SNARE proteins in distinct intracellular pathways. The specificity of intracellular membrane trafficking and fusion processes depends on both, SNARE proteins and SM proteins (Scales et al., 2000; Toonen and Verhage, 2003). While approximately 30 different SNARE proteins are known in mammals, only 4 distinct SM proteins groups are identified (fig1.2; for review see (Hong and Lev, 2014)). SM proteins bind to individual SNARE proteins or assembled SNARE complexes (Dulubova et al., 2007) (for review see (Südhof and Rothman, 2009)).

SNARE complexes and SM proteins in vesicle biogenesis

The biogenesis of secretory vesicles is a highly complex mechanism that includes a number of SNARE-dependent steps. Distinct SNARE complexes are formed when carrier vesicles with precursor peptides from the ER fuse with the membrane of the ER-Golgi intermediate compartment and subsequently with the membrane of the *cis*-Golgi. The earliest stages are hereby regulated by a SNARE complex composed of Syntaxin5, GS27, Bet1 and Sec22b (Hay et al., 1998). A SNARE complex consisting of Syntaxin5, Bet1, GS28 and Ykt6 is implicated in the late stages of ER to Golgi transport mechanisms (Zhang and Hong, 2001), whereas a complex with a very similar composition (Syntaxin5, GS15, GS28 and Ykt6) mediates transport processes between Golgi cisternae (Xu et al., 2002).

Retrograde transport from the Golgi apparatus to the ER is also SNARE dependent and recent studies identified a quaternary SNARE complex, consisting of mSec22b, mUse1/D12, mSec20/BNIP1, and Syntaxin18 (Verrier et al., 2008). The SM protein SLY1 is involved in Syntaxin5-dependent transport processes between ER and Golgi (Rowe et al., 1998), as well as in intra-Golgi transport and retrograde transport (Laufman et al., 2008). After the budding of ISVs from the TGN, a different set of SNARE proteins and SM proteins is necessary for further maturation steps. SNARE proteins that are specifically localized on the membrane of ISVs, but are not found on MSVs are believed to be involved in the maturation process of secretory vesicles (*e.g.*, Syntaxin6 (Klumperman et al., 1998) and vesicle associated membrane protein (VAMP) 4 (Steegmaier et al., 1999)). Syntaxin6 is a core component of the molecular machinery involved in homotypic fusion of ISVs (Wendler et al., 2001). VAMP4 recruits AP-1, which is essential for the removal of excess membrane during vesicle maturation (Peden et al., 2001; Hinners et al., 2003). During the maturation of ISVs to MSVs, VAMP4 is sorted away from the ISV membrane (Eaton et al., 2000). VAMP4 is no longer necessary after the sorting pathway is initiated. Therefore it needs to be removed from the ISVs in order to become MSVs that are responsive to increased Ca^{2+} levels. Even though the general pathway of secretory vesicle biogenesis and maturation is known, molecular details remain to be discovered.

SNARE complexes and SM proteins in regulated secretion
The function of the SNARE complex and the SM protein Munc18-1 during exocytosis were studied extensively (for recent reviews see (Südhof and Rothman, 2009; Südhof, 2013)). As described above, the cascade of events during secretion involves the docking of secretory vesicles to the PM, the priming of the vesicle in order to gain fusion competence and the actual fusion of the membranes. The PM-localized SNARE proteins Syntaxin1a and SNAP-25 and the SM protein Munc18-1 are crucial for the docking process (Voets et al., 2001; de Wit et al., 2006). During the docking process Munc18-1 is bound to Syntaxin1a in its "closed" confirmation (Hata et al., 1993; Dulubova et al., 1999). The actual fusion process of the PM and the secretory vesicle membrane requires the formation of the four-helical SNARE complex consisting of PM localized Syntaxin1a, SNAP-25 and VAMP2 at the membrane of the secretory vesicle. To form a SNARE complex with SNAP-25 and VAMP2, Syntaxin1a has to change its confirmation from "closed" to "open" (Dulubova et al., 1999; Misura et al., 2000). The zippering of the four-helical bundle in N-to-C-terminal direction generates the force necessary for membrane fusion (Walter et al., 2010).

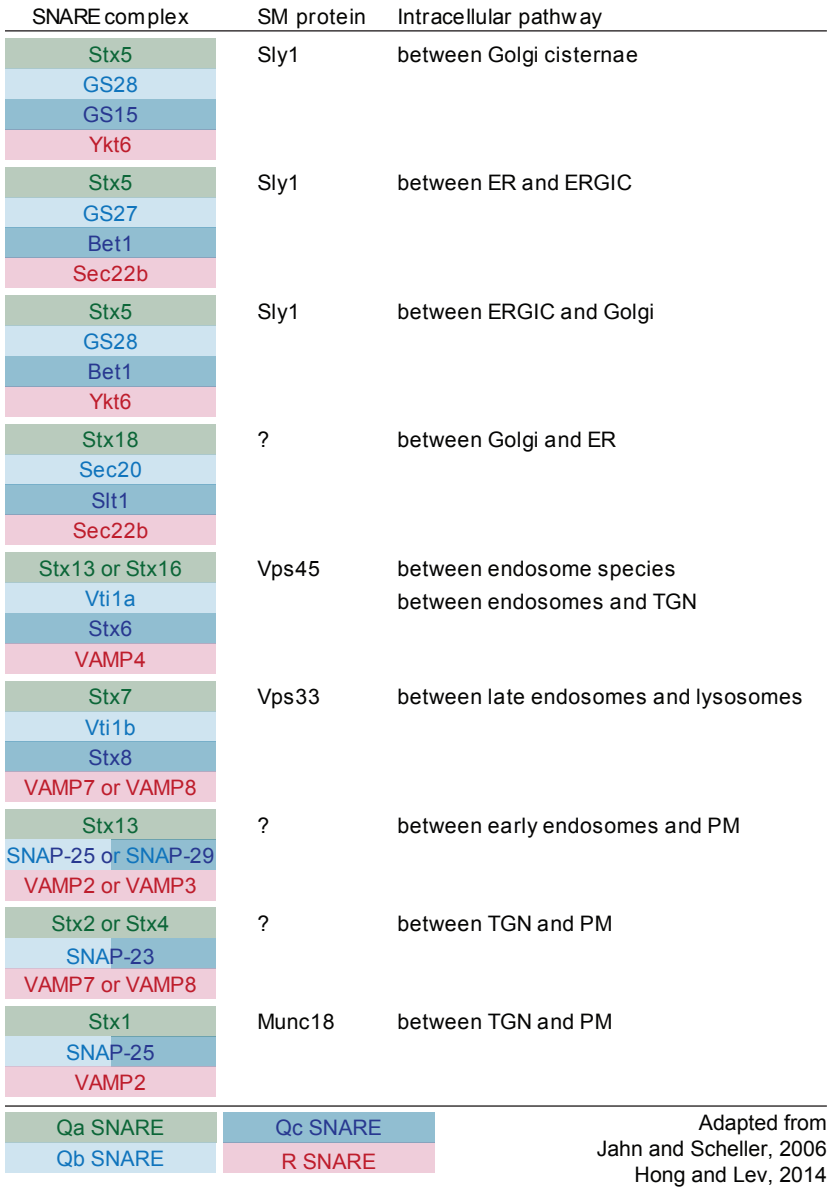

Figure 1.2: Overview of SNARE complexes and corresponding SM proteins in intracellular pathways in mammalian cells: SNARE complexes are formed by three or four SNARE proteins and consist of four α-helices.

Cytoskeleton

The cytoskeleton in CCs includes microtubules, myosin and actin filaments. The microtubules are primarily localized in the vicinity of the TGN, whereas myosin and actin are primarily found in cortical regions (Aunis and Bader, 1988). Actin filaments form a dense network underneath the PM.

Cytoskeleton in vesicle biogenesis
Cytoskeleton components play a role during maturation processes of secretory vesicles. The directional transport of ISVs from the TGN to the PM is microtubule-dependent (Rudolf et al., 2001). It was shown that the majority of ISVs is locally restricted in the cortical F-actin network, where ISVs move in a Myosin Va-dependent manner (Kögel et al., 2010). Maturation processes, such as homotypic fusion, of ISVs occur in the cortical F-actin network (Rudolf et al., 2001).

Cytoskeleton in regulated secretion
Two systems of cytoskeletal components influence regulated exocytosis in distinct ways. The application of microtubule inhibitors results in a 20 % reduction of the slow phase of secretion, without significantly effecting the burst phase (Neco et al., 2003). This finding is consistent with a role of microtubules during the transport of ISVs to the PM.

The Actin-Myosin system is the dominant cytoskeleton system during regulated exocytosis of secretory vesicles in CCs. Inhibition of this system results in a strong decrease in the slow phase and in the burst phase of secretion (Neco et al., 2003). Myosin Va was suggested to regulate the transport of secretory vesicles from the reserve pool to the RRP (Lejen et al., 2003; Watanabe et al., 2005). The cortical F-actin network has been proposed to play a dual role in regulated exocytosis: (1) as negative regulator of exocytosis by functioning as storage for secretory vesicles under resting conditions and as physical barrier for secretory vesicles approaching release sites at the PM (Eitzen, 2003; Malacombe et al., 2006; Gutiérrez, 2012) and (2) as active facilitator of secretory vesicle transport to the PM and of fusion processes at release sites (Sokac and Bement, 2006; Nightingale et al., 2012). The idea of the cortical F-actin network as vesicle storage is based on the attachment of vesicles to actin filaments and the observation that vesicles detach from the cytoskeleton upon stimulation. The model of actin as physical barrier is based on the observation that the disruption of the F-actin network leads to an increase in motility of secretory vesicles and enhanced exocytosis (Miyamoto, 1995; Giner et al., 2005). Actin also plays a role in the facilitation of active transport of secretory vesicles. These transport processes along actin filaments require actin-based motors, such as myosin family members (Rudolf et al., 2003). Both roles of actin are equally important, since it was shown that a partial disruption of the cortical F-actin network results in increased exocytosis, whereas total disruption leads to a total block of secretion. Therefore a highly organized actin regulation is crucial for exocytosis in CCs.

Lipid metabolism

Lipids are the main components of biological membranes and function as active signaling molecules during vital cellular processes such as biogenesis (Siddhanta and Shields, 1998; Huttner and Zimmerberg, 2001) and exocytosis (Di Paolo and De Camilli, 2006; Martin, 2012; Ammar et al., 2013). Especially phosphoinositide (PI) species play a regulatory role in most cellular processes (for review see (Vicinanza et al., 2008; McCrea and De Camilli, 2009; Idevall-Hagren and De Camilli, 2015). The PI metabolism underlies a strict spatio-temporary control, which is based on compartmentalization and a complex system of enzymes such as kinases, lipases and phosphatases (fig1.3 and fig1.4).

Lipids in vesicle biogenesis
Cholesterol, sphingomyelin and other lipids form microdomains in the Golgi membrane. Those lipid rafts constitute the localization from where ISVs bud from the TGN (Dhanvantari and Loh, 2000). The second messenger DAG and phosphatidic acid (PA) play an important regulatory role during the budding of secretory vesicles from the TGN (Kearns et al., 1997; Antonny et al., 1997; Litvak et al., 2005; Chen and Shields, 1996). The insertion of those cone-shaped lipids (DAG, PA) induces negative membrane curvature, which facilitates semi-fusion intermediates and stimulates membrane fusion (Burger, 2000). Furthermore, DAG recruits a number of signaling proteins (*e.g.*, protein kinase D (Baron and Malhotra, 2002; Yeaman et al., 2004), protein kinase C isoforms (Carrasco and Merida, 2004) and ADP ribosylation factor (Arf) GTPases (Yanagisawa et al., 2002)) to the Golgi apparatus. Protein kinase D activates PI4KIIIβ (Hausser et al., 2005), which generates PI(4)P by the phosphorylation of PI (Weixel et al., 2005). PI(4)P recruits several proteins to the Golgi membranes (D'Angelo et al., 2008). One example is adaptor protein (AP-1), which plays an important role in vesicle biogenesis and maturation, but also regulates the transport of secretory vesicles to the PM (Mills et al., 2003; Godi et al., 2004). Another study proposed a contribution of Golgi-localized PI(4)P to the pool of PI(4,5)P_2 at the PM and thereby also connects biogenesis and regulated exocytosis (Dickson et al., 2014).

Lipids in regulated secretion
The lipid composition of the PM determines the action of the exocytosis machinery (for reviews see (Ammar et al., 2013; Martin, 2012)). Lipids are not homogenously distributed in the PM, but form microdomains that are enriched in specific lipids (Laux et al., 2000). Those clusters mark release sites and are often enriched in lipids such as cholesterol (Lang et al., 2001) and PI(4,5)P_2 and strongly co-localize with secretory vesicles attached to the membrane (Aoyagi et al., 2005). Besides the spatial determination of fusion sites, PI(4,5)P_2 also recruits proteins that regulate exocytosis (James et al., 2008). Especially PI(4,5)P_2 was shown to recruit the docking factor Synaptotagmin1 (Syt-1)(Schiavo et al., 1996) and the priming factors CAPS (Loyet et al., 1998) and Munc13 (Shin et al., 2010) to release sites at the PM.

Recently it was shown that $PI(4,5)P_2$ also regulates the function of ion channels, such as high-voltage gated Ca^{2+} channels (Suh et al., 2010) and K^+ channels (Whorton and MacKinnon, 2011). $PI(4,5)P_2$ indirectly influences exocytosis via its role in the regulation of cytoskeletal proteins. $PI(4,5)P_2$ activates or inhibits actin binding proteins and thereby regulate the organization and dynamics of the actin cytoskeleton in multiple ways (for review see (Sechi and Wehland, 2000; Janmey and Lindberg, 2004)). First, $PI(4,5)P_2$ binds and activates proteins that are involved in membrane linkage of actin filaments, *e.g.*, vinculin (Niggli et al., 1986; Ito et al., 1983) or α-actinin (Burn et al., 1985). Second, it initiates actin assembly and nucleation via its interaction with the actin-related protein (Arp) 2/3 complex and Wiskott-Aldrich syndrome protein (WASP, (Miki et al., 1999)). Third, $PI(4,5)P_2$ activates actin-crosslinking proteins, such as myristoylated alanine-rich protein kinase C substrate (MARCKS (Glaser et al., 1996; Laux et al., 2000)) and it inhibits filament severing proteins such as scinderin (Zhang et al., 1996) and cofilin (van Rheenen et al., 2007). In general, an increase of PM localized $PI(4,5)P_2$ results in actin assembly and stabilization, whereas a decrease primarily leads to actin disassembly (for review see (Janmey and Lindberg, 2004). As described earlier, those highly regulated actin dynamics are proposed to play a role in regulated secretion. Lipids play a regulatory role in biogenesis and regulated exocytosis. Protein recruitment and the determination of microdomains with specific functions are essential lipid-dependent requirements in both processes.

Only when one fully understands the functional synergy between those three cellular components ((1) SNARE proteins and SM proteins, (2) Cytoskeleton, (3) Lipids) one will be able to grasp the events in the life of a secretory vesicle in adrenal chromaffin cells.

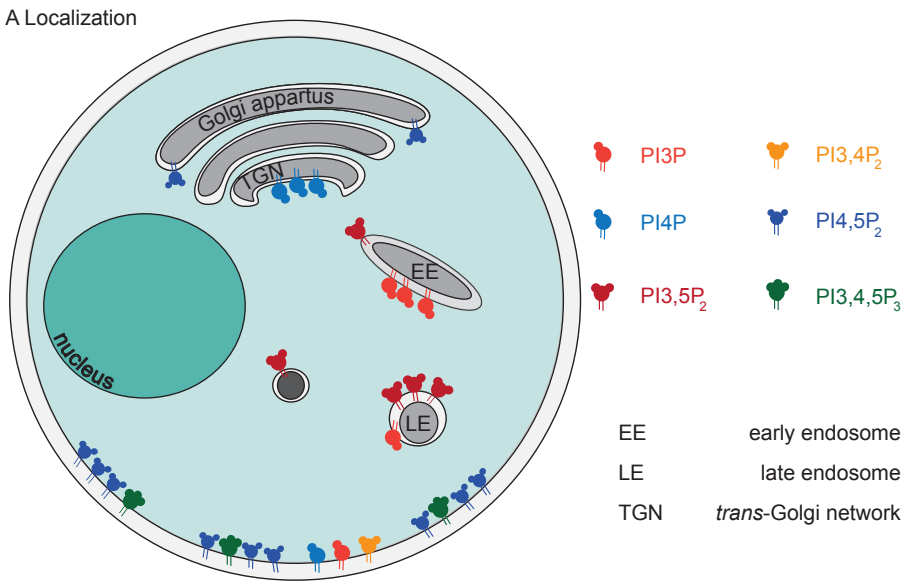

Figure 1.3: Overview of phosphoinositide species (A): Primary localization of six different PI species in adrenal chromaffin cells; adapted from (Vicinanza et al., 2008; McCrea and De Camilli, 2009; Idevall-Hagren and De Camilli, 2015))

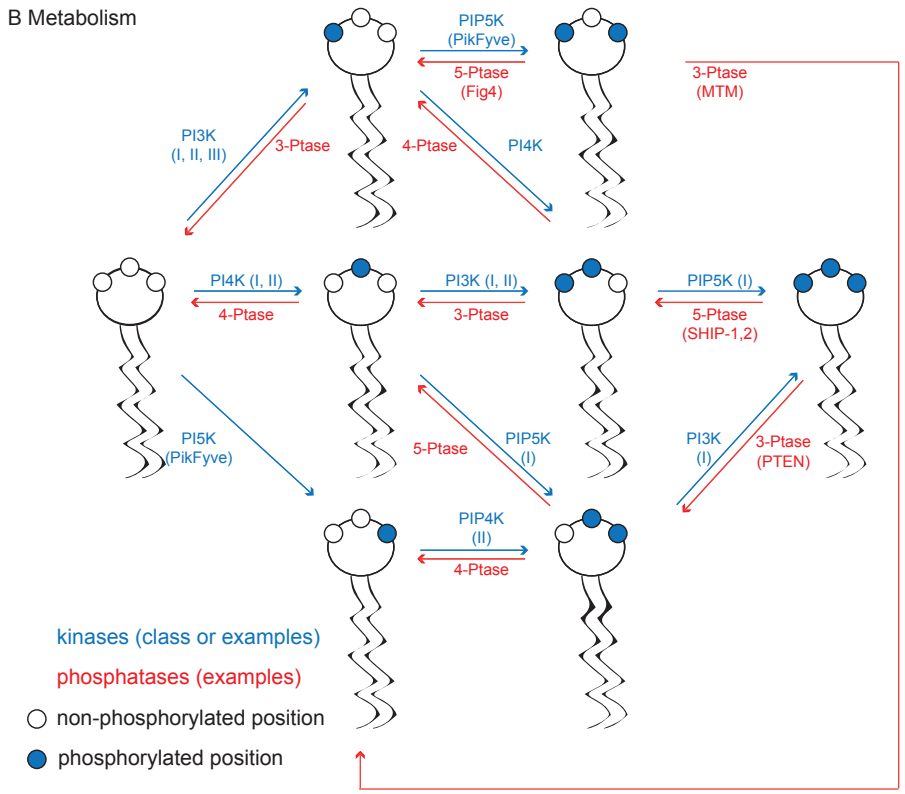

Figure 1.4: Overview of PI metabolism (B): General PI metabolism; shown are eight different PI species (empty circles indicate phosphorylatable positions, blue circles indicate phosphorylation on position 3, 4 or 5) and phosphorylation (blue) or de-phosphorylation (orange) steps; adapted from (Vicinanza et al., 2008; McCrea and De Camilli, 2009; Idevall-Hagren and De Camilli, 2015))

Aim and Outline

The general aim of this thesis was the expansion of our knowledge of molecular mechanisms that are critical in regulated secretion of large dense-core vesicles in CCs: from the biogenesis of vesicles at the ER and the Golgi apparatus to their final destination at release sites at the PM.

In **Chapter 2** of this thesis, we identified the SNARE protein Vti1a as an important factor in large dense-core vesicle biogenesis in CCs. We showed that the absence of *vti1a*, but not *vti1b*, resulted in impaired secretion due to a severe reduction of large dense-core vesicles. We were able to rescue the phenotype by the re-introduction of *vti1a*.

In the following chapters we focused primarily on regulated exocytosis and the involvement of the cortical F-actin network in this process. In order to automatically analyze F-actin alterations due to genetic modifications or cell stimulation, we developed an analysis algorithm ("PlasMACC"), which enables us to quantify changes in fluorescent signals at the PM. The algorithm and several application examples are presented in **Chapter 3**.

Based on its dual function, the cortical F-actin network is highly dynamic and regulated by a multitude of factors. In **Chapter 4**, we investigated a F-actin mediating role of the SM protein Munc18-1, which is primarily known for its function in docking and secretion. As shown before, the absence of *munc18-1* in CCs resulted in an increased subplasmalemmal F-actin network. In order to further characterize Munc18-1s function in F-actin regulation, we expressed homologues, orthologues and mutants to pinpoint specific residues or domains that are critical in this process. Mutations in domain 3, specifically on residue V263 resulted in Munc18-1 mutants, which were no longer able to regulate the cortical F-actin network.

In **Chapter 5**, we studied whether the Munc18-1 mediated F-actin regulation is attributable to the phosphoinositide $PI(4,5)P_2$, which is known to control a wide variety of actin-regulating proteins. We therefore compared the levels of $PI(4,5)P_2$ at the plasma membrane in the presence and absence of Munc18-1 and we found an increase in CCs from *munc18-1* null mice.

The main findings of these studies are summarized in **Chapter 6** of this thesis. We hereby integrated our discoveries in the already comprehensive network of proteins, lipids and cell organelles, which are involved in the process of regulated secretion in CCs. Based on the literature and our findings, we develop molecular models and hypotheses which need to be tested in the future.